EXTENT OF CHROMOSOMAL DAMAGE BY AGRICULTURAL PESTICIDES IN *RATTUS NORVEGICUS*

DISSERTATION

SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS
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IN

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(GENETICS)

By

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CERTIFICATE

This is to certify that the dissertation entitled **" Extent of chromosomal damage by agricultural pesticides in *Rattus norvegicus*"** embodies the original research work independently pursued by **Mr. Mohammad Faisal Siddiqui** under my supervision. I have permitted him to submit it towards the partial fulfillment of the requirement of the degree of Master of Philosophy in Zoology.

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Abbreviations Used:

b.wt.	Body weight
CA	Chromosomal aberration
CAA	Chloroacetic Acid
CB	Chlorobenzene
CDRI	Central Drug Research Institute
DH_2O	Distilled water
DNA	Deoxyribose Nucleic Acid
FBS	Fetal Bovine Serum
g	gram
h	hour
i.p.	Intraperitoneal
LD_{50}	Lethal median dose or Median lethal dose
MCA	Monochloroacetic Acid
MCB	Monochlorobenzene
mg	milligram
min	minute
MN	micronuclei
$MNNCE_S$	Micronucleated Normochromatic Erythrocytes

$MNPCE_S$	Micronucleated Polychromatic Erythrocytes
MNT	Micronucleus test
NCE_S	Normochromatic Erythrocytes
PCE_S	Polychromatic Erythrocytes
RNA	Ribose Nucleic Acid
TCA	Trichloroacetic Acid

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-Mohammad Faisal Siddiqui

INTRODUCTION

General review and justification of the proposal:

The beginning of the genetic toxicology was marked by H.J. Muller's discovery in 1927 that X-rays cause sex-linked recessive lethal mutations in *Drosophila*. Later on, mutagenic properties of chemical compounds were appreciated by Oehlkers (1943) and Auerbach and Robson (1946). The importance of mutation studies were recognized by Alexander Hollaender (1960's) who founded Environmental Mutagen Society (EMS) in 1969, in USA (Hoffman, 1966). The prominent among these societies are: International Association of Environmental Societies (IAEMS), National Institute of Health (NIH), National Toxicology Program (NTP), Environmental Protection Agency (EPA), Food and Drug Administration (FDA), which have supported the basic research in mutagenesis. The U.S. Environmental Protection Agency (EPA) under its Gene-Tox Program has developed a large number of genetic bioassays (Brusick and Auletta, 1985). The coordinated attempts under the auspices of these societies enhanced research on toxicity taking into account specific concern to human health and emphasis on changes in DNA. Consequently, "genotoxicity" as a discipline was established.

We now broadly define genotoxicity as systematic investigations on the effects that any physical or chemical agents present in our environment can exert at subtoxic levels on the genes or other nucleotide sequences in a genome of an organism, which ultimately affect gene function. The objective of investigations on genotoxicity should, therefore, be: (i) direct changes induced in the genetic material (DNA); (ii) elucidation of mechanisms involved and (iii) effects at the level of gene pool (Jha, 1998). A primary reason for this complexity is the multistep nature of inducible mutations and /or carcinogenicity including both genetic and non-genetic (metabolic) events (Harris, 1991).

As pointed out in the previous paragraph, any chemical, physical or biological agent can be classified as genotoxic if it induces alterations in nucleic acids and associated components at sub-toxic exposure level and modify hereditary characteristics of DNA. Some of the agents under this category are redefined as clastogens when they induce chromosome breaks permitting observations at metaphase. Since chromosomal breaks or rearrangements (chromosomal aberrations=CAs) are actually the result of unrepaired DNA damage and it has to be significant.

Certain aspects of genotoxicity of a number of pesticides in plants and

animals have already been worked out (Nygren, 1949; Wuu and Grant, 1966; Hollaender, 1971; Majumdar and Hall, 1973; Darving and Hultgren, 1977; Bhunya and Behera, 1984). Pesticides, insecticides and fumigants, which are extensively used in modern agriculture are of immediate concern to man (WHO, 1972). Available literature provides a window into health and chemical safety evaluation on genotoxic chemical contaminants (Moutschen, 1985; Hoffman, 1966), while their levels continue to rise in our environment. Irrevocable evidence exists which shows that it is damage to DNA caused by these agents, which results in somatic as well as heritable mutations (Bridges, 1976; ICPEMC, 1983; Russell and Shelby, 1985; Shelby, 1988). It is not thus surprising that a number of them known mutagens, carcinogens and teratogens.

A variety of testing protocols have been developed which extend the analysis to permit evaluation of mutagenesis including clastogenic properties of various chemical and physical agents (Fishbein *et al.*, 1970; Legator, 1970; Hollaender, 1971). Ames' test has been widely employed to detect chemical mutagenesis (Ames, 1971; Ames *et al.*, 1975; Ames, 1979). Besides this test for primary screening, other assay systems such as detection of the ability of chemicals to cause chromosomal breakages have been widely in use (Karkaya *et al.*, 1997; Choudhary *et al.*, 2000; Thust *et al.*,

2000). The list includes routine molecular techniques, chromosomal banding or fluorescence *in situ* hybridization (FISH) (Fearon & Vogelstein, 1990; Sandberg, 1993).

A number of *in vivo* and *in vitro* studies have used the above approaches to show cytogenetic alteration which can result from exposure to various chemical mutagens and ionizing and non-ionizing radiations (Lucas *et al.*, 1992; Zeeb and Blettner, 1998; de Gruijl, 2002). Chromosomal aberrations (CAs) contribute significantly to genetic disease-burden in humans. Specific CAs have been reported in congenitally defective children. Many tumors in mammals (including human beings) show an altered karyotype and chromosomal changes have been implicated in *neoplasia* (Radman *et al.*, 1982). Chromosomal changes associated with particular tumor types have been comprehensively classified by Mitelman (1988; 1994) and Heim & Mitelman (1995). To increase the sensitivity of the protocols for detecting mutagenic effects, cell lines with defects in DNA repair have been developed (Evans *et al.*, 1979; Allen *et al.*, 1986). In almost all studies, structural and/ or numerical chromosome alterations were a rule. Thus, chromosomes are sensitive enough to respond to any insult, which facilitates its detection (Solomon and Goddard, 1991; Mitelman, 1994; Rabitts, 1994; Choudhary *et al.*, 2000; Barton *et al.*, 2003; Watanabe, 2003).

Observations on CAs and MN formation are an appropriate assay for clastogenicity and in turn, reliable predictors of carcinogenicity, because these changes are the result of direct damage inflicted by the mutagen.

Review on Employed Cytogenetic Assays

The two cytogenetic assays, which have been employed during the present study, are:

i) Chromosomal aberration

ii) Micronucleus test

These assays are recognized for predicting mutagenicity, clastogenicity and carcinogenicity of various chemicals.

Chromosomal Aberration Assay

The Ad-hoc Committee of the Environmental Mutagen Society and the Institute of Medical Research has provided a classical protocol using bone marrow from mice, rats, or hamsters for Medical Research (1972). The mammalian *in vivo* test is used for the detection of structural chromosome aberration by the test substances to the bone marrow cells of animals, usually rodents (Adler, 1984; Preston *et al.*, 1987; Richold *et al.*, 1990; Tice

et al., 1994). The most readily detected aberrations with the greatest accuracy were asymmetrical exchanges such as dicentrics and rings, and terminal and interstitial deletions (Savage, 1976). These events lead to loss of chromosomal material at mitosis, or they inhibit accurate chromosome segregation at anaphase. All types of chromatid aberrations are detectable in chromosomes, and many of these events lead to cell killing through loss of chromosomal DNA. The use of metaphase chromosomes in mammalian cells to evaluate chromosome damage has gained wide application because of the ease by which chromosomes can be spread with hypotonic solution, while spindle fiber formation can be blocked with colchicine (NIOSH, 1989). Development of mammalian cell culture techniques has further facilitated these studies. Metaphase aberrations have been studied both to detect genetic changes in cancer cells and to quantitate the amount of environmentally induced genetic damage in cells of humans and experimental animal populations (Ashby, 1983; Lucas *et al.*, 1992; Ashby, 1986; Zeeb and Blettner 1998; Antonelli *et al.*, 2003).

Micronucleus Test Assay

The bone marrow micronucleus test (MNT) was first developed by Bollar and Schmid (1970) as a simple assay system for cytogenetic analysis. Bone

marrow cells are most commonly used targets in *in vivo* studies. A number of subsequent reports demonstrated the potential of using bone marrow micronuclei for evaluating the clastogenicity of several chemical substances (Matter and Schmid, 1971; Heddle, 1973; Schmid, 1975, 1976; Heddle *et al.*, 1983; Choy *et al.*, 1985; MacGregor *et al.*, 1980; MacGregor, 1987; Choy *et al.*, 1993; Ateeq *et al.*, 2002; Iton *et al.*, 2002). This is however, evident that the micronucleus *in vivo* test is a method primarily meant to screen chemicals for chromosome-breakages (Von Ledebur and Schmid, 1973; Schmid, 1976; Salamone and Heddle, 1983). Micronuclei can be found in myeloblasts, myelocytes and erythroblasts (Schmid, 1975). In cell types with little cytoplasm, they are not always easily distinguishable from normal nuclear lobes or projections (Schmid, 1975). Later on, mouse bone marrow MNT gained a wide acceptability in mutagenicity testing and approved by legislative and international agencies (Yamamoto and Kikuchi, 1980).

Review of the Literature on Chlorobenzene and Chloroacetic Acid

Chlorobenzene *:

Chlorobenzene (CB) is one of the twelve (12) compounds in the group of chlorinated benzenes. CB is a colorless, flammable liquid used as additives, as an industrial solvent and chemical intermediate with numerous process applications in pharmaceutical industry. In production of pesticides using CB as an intermediate has declined and no new report on CB has appeared in recent years. CB is produced commercially by the chlorination of benzene in the presence of a catalyst (*e.g.* ferric chloride, aluminium chloride, or stannic chloride). This process yields a mixture of chlorobenzene, dichlorobenzene and higher analogs which are distilled and crystallized to obtain pure products (Hayes *et al.*, 1973). It is also used in the synthesis of organochlorine pesticides, including DDT, as well as phenol, picric acid and dyes. It is now used primarily as a degreasing solvent, as a chemical intermediate in the synthesis of nitro-chlorobenzenes, in the dry cleaning industry and in the manufacture of pesticides, resins, dyes and perfumes. As obvious, chlorobenzene has no natural source but it accumulates in fish, aquatic invertebrates and algae from the above practices and industrial sources (U.S. EPA, 2001; U.S. EPA, 2003).

Studies on animals show that chlorobenzene is lethal following acute, intermediate and chronic oral exposure (Hayes *et al.*, 1973; Davis and Bernt, 1987; Bhat *et al.*, 1991; Bryant *et al.*, 1992; NTP, 1992). Death occurs within 2-3 days after a single exposure to 4,000 mg / kg in corn oil by gavage to rats of both sexes and in mice after a single exposure to 1,000 mg / kg (NTP, 1985). In a 14 day repeated dose gavage study, administration of 1,000 mg / kg was lethal to all rats by the end of study (NTP, 1985). The acute toxicity of CB is relatively low and mainly associated with acute central nervous system (CNS) effects (Kluwe *et al.*, 1985; Hellman, 1992). Repeated administration of CB to experimental animals for several weeks or months, orally or by inhalation, have been associated with toxic effects in liver and kidneys (Kluwe *et al.*, 1985; Hellman, 1992). Moreover, after repeated inhalation of the compound, mice were reported to develop leukopenia and a general bone marrow depression (Zub, 1978).

The results of testing genotoxicity and mutagenicity potential of CB in various test systems are not consistent (Hellman, 1992). Although the majority of *in vitro* system test results are negative, high concentrations of CB have been reported to induce gene mutation in L5178Y mouse cell lymphoma assay (McGregor *et al.*, 1988), as well as chromosomal aberration in the MNT employing bone marrow cells from mice

(Mohtashamipur *et al.*, 1987). The toxicity of chlorobenzene is mediated by a metabolic product, presumably an epoxide (Williams, 1959; Brodie *et al.*, 1971; Reid *et al.*, 1971; Reid, 1973; MacGregor *et al.*, 1990).

In the previous studies, it was found that chlorobenzene differs from the genotoxic cytostatic agent cyclophosphamide in lacking DNA damaging effects in bone marrow cells, even after repeated high dose exposure (Vaghef and Hellman, 1995).

However, since CB affected the DNA in the peripheral lymphocytes, it was concluded that this compound has a moderate genotoxic potential when administered in high doses *i.e.*3×750 mg/kg (Vaghef and Hellman, 1995). The observation of a DNA-damaging potential of CB conforms with a recent report of an increased frequency of gene mutations in peripheral lymphocytes from human with several years of occupational exposure to the compound (Major *et al.*, 1993). These findings suggest that CB represents a genotoxic hazard at high sub- lethal doses, a view supported by experiments showing that metabolites of CB can bind to nucleic acids, both *in vivo* and *in vitro* in mice and rats (Grilli *et al.*, 1985; Prodi *et al.*, 1986; Colacci *et al.*, 1990). The Physico-chemical properties of CB and its metabolic pathway are given below:

Physico-chemical properties

Chlorobenzene

Chlorobenzene	
PROPERTIES	**VALUE**
Common information:	
Common name	Chlorobenzene
IUPAC name	Monochlorobenzene
Chemical class	Chlorinated aromatic compound
CAS. R No.	108-90-7
Physico-chemical properties:	
Molecular formula	C_6H_5Cl
Molecular weight	112.56 gm/mol
Appearance	Colorless liquid
Melting point	-45.6^0C
Boiling point	132^0C
Solubility	Water 500 mg /L @20^0C; soluble in organic solvents e.g., alcohol ether, benzene.
Density	1.1058
Mammalian toxicity:	
Acute	Intraperitoneal LD_{50} 16.6 mg/kg for rats
Ingestion	Harmful if swallowed

* Source : (http://www.epa.gov/iris/subst/0399.htm).

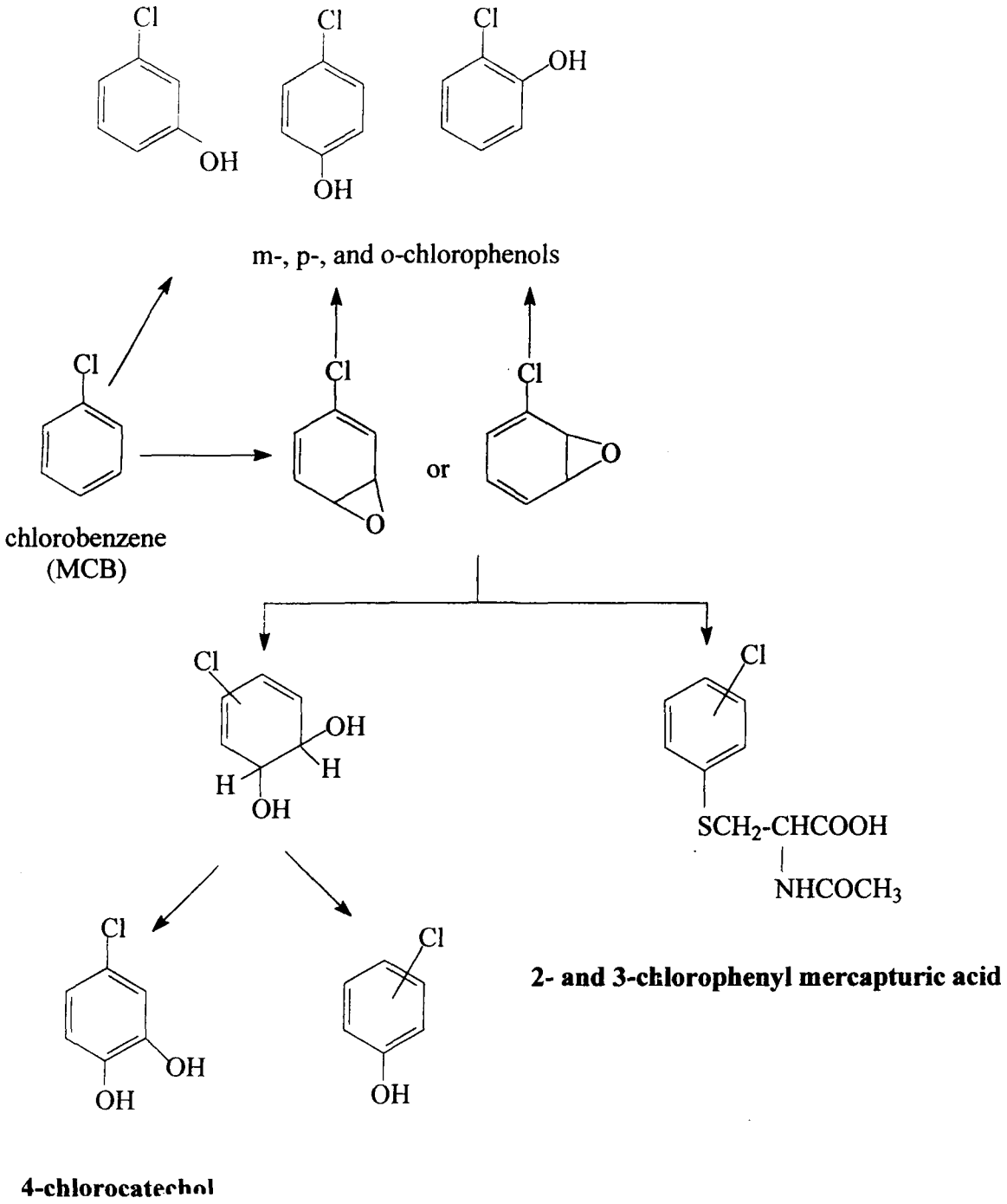


Figure 1. Main metabolic pathways of chlorobenzene (Ogata and Shimada, 1983).

The study of potential genotoxicity of chlorobenzene was analyzed using a rapid and visual method for detecting DNA damage in individual mammalian cells. Whereas bone marrow cells were chosen as potential targets for CB-induced toxicity and genotoxicity, peripheral blood lymphocytes were taken as easily accessible cells (Vaghef and Hellman, 1995).

Chloroacetic Acid:

Chloroacetic acid (CAA) is used as a herbicide and preservative, as a treatment for plantar warts, as a drying agent for curing hay and as a chemical intermediate in production of several other chemicals. These include carboxymethyl cellulase, ethyl chloroacetic acid, glycine, synthetic caffeine, sorcosine, thioglycolic acid, EDTA, the herbicides 2,4-D and 2,4,5-T and vitamin A (U.S. EPA, 1988).

Thousands of industrial and agricultural workers are exposed to CAA. CAA is also produced in the environment as one of the metabolites of other widely used chemicals like vinyl chloride, vinylidene chloride, 1,1,2-trichloroethane and 1,2-dichloroethane (Yllner, 1971; Bartsch *et al.*, 1976; Hathway, 1977). CAA can also form as a by-product of mustard (Agent H) degradation products and decontamination materials such as hypochlorite

(Woodard *et al.*, 1941; Chenoweth, 1949; Webb, 1966; Sittig, 1985; U.S. EPA, 1988; Budavari, 1989; NTP, 1992). CAA is also known to be one of the most commonly detected disinfection by-products in the drinking water supply of the United States (Christman *et al.*, 1983; Norwood *et al.*, 1983; Uden and Miller, 1983; Krasner *et al.*, 1989; Plewa *et al.*, 2002).

Detailed information on CAA will guide the health-care professionals in treatment of patients accidentally exposed to potentially lethal dose of CAA (Bernard *et al.*, 1991). Physico-chemical properties and composite metabolic pathway of CAA are given on the page No. 16 and 17.

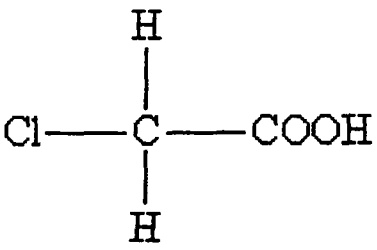
Chloroacetic acid is rapidly and efficiently absorbed through the skin. It is not only highly corrosive to tissues topically but also can cause death systematically after various routes. Even as little as 1% exposure to skin can be harmful and individual has to be hospitalized (Mann, 1969; Quick *et al.*, 1983; Kusch *et al.*, 1990; Kulling *et al.*, 1992). Several reports are available wherein cases of accidental poisoning have been found to be fatal to humans and animals (Zeldenrust, 1951; Christiansen and Dalgaard–Mikkelsen, 1961; Mann, 1969; Quick *et al.*, 1983; Kusch *et al.*, 1990; Kulling *et al.*, 1992). There are many studies describing the acute, subchronic and chronic toxicity of CAA (Hayes *et al.*, 1973; Davis and Bernt, 1987; Bhat *et al.*, 1991;

Bryant *et al.*, 1992; NTP, 1992; Nelson *et al.*, 2001; Shakil and Rozman, 2003), which suggest the lack of mutagenicity (Rannug *et al.*, 1976) and its carcinogenic potential (Innes *et al.*, 1969; Van Duuran *et al.*, 1974; NTP, 1992).

Previous reports indicated that CAA was acutely toxic and cause neurological abnormalities in geese (Christiansen and Dalgaard-Mikkelsen, 1961), cattle and sheep (Quick *et al.,* 1983). There are at least two reports of human death associated with CAA exposure (Zeldenrust, 1951; Mann, 1969).

Physico-chemical properties

Chloroacetic acid



Chloroacetic acid

PROPERTIES	VALUE
Common information:	
Common name	Monochloroacetic acid or MCA
IUPAC name	Chloroacetic acid
Chemical family	Halogenated aliphatic carboxylic acid/haloalkanoic acid /chlorinated carboxylic acid
CAS. R No.	79-11-8
Physico-chemical properties:	
Molecular formula	$CH_2ClCOOH$
Molecular weight	94.5 gm/mol
Appearance	White crystalline solid
Melting point	62-64°C
Boiling point	102°C
Solubility	Aqueous solution and soluble in ethanol, benzene. chloroform and diethyl ether.
Stability	Stable; at least 7 day @ 25°C; 32 day refrigerated
Vapor Density	3.25 (air =1)
Partition coefficient:	
Vapor pressure	0.1Kpa (0.75mm Hg)@ 20°C
Mammalian toxicity:	
Acute	Oral LD_{50} 76mg / Kg for rat oral LD_{50} 165 mg / kg for mouse; intraperitoneal 16600 µg/kg for rats
Ecotoxicity	Bird - toxic to poultry, Fish – LC_{50} (48h) for rainbow trout 900 mg/L, Bees – toxic to bees

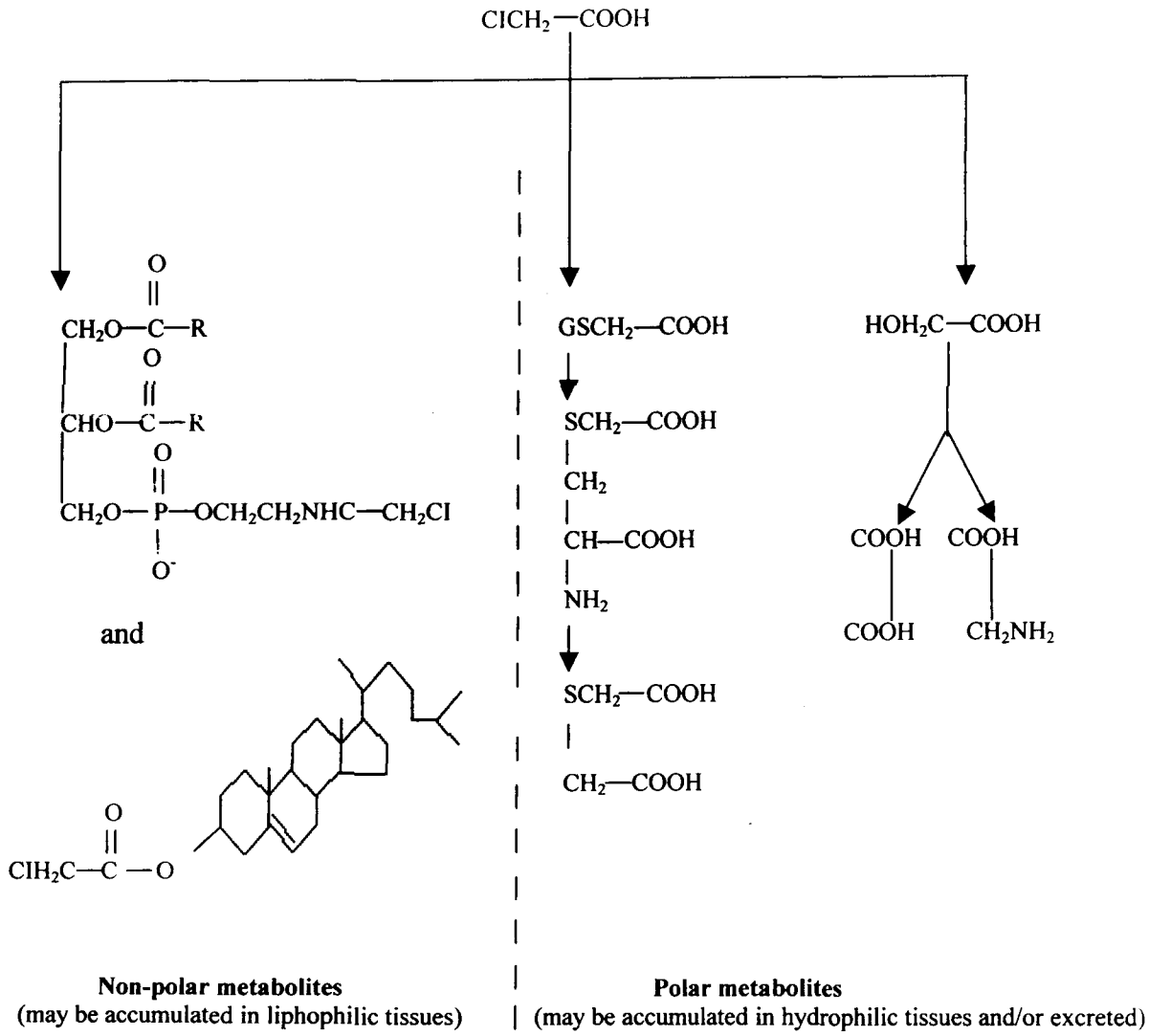


Fig. 2. Composite metabolic pathway of Chloroacetic acid (Bhat *et al.*, 1990)

OBJECTIVES

Taking into account, particular concerns about possible mutagenic properties of substances that regularly enter in human body (*e.g.* pesticides, drugs, food additives, sprays, chlorinated hydrocarbons), it is important to evaluate genotoxicity by using mammalian models. The proposed dissertation was planned keeping in mind that available information on chloroacetic acid (CAA) and chlorobenzene (CB) chosen here needs further investigation. Summary of the physiochemical properties and brief toxicology of these compounds is given in Literature review on CB and CAA (page No. 11 and 15). The following objectives were set:

1. To study mutagenicity of CB and CAA by using *Rattus norvegicus* as mammalian model.

2. To study the mechanism of action and effects of CB and CAA on bone marrow cells of *Rattus norvegicus* by applying the *in vivo* assay systems.

The review of Cytogenetic Assays employed in this study and Physico-chemical properties and metabolic pathway of CAA and CB have already been described in the previous page No. 11 and 16.

MATERIALS AND METHODS

Chemicals and Reagents:

Chemicals and other routine reagents were of high quality and purity grade and available commercially. Some of them are listed below:

Source:

Chemical	**Source**
Acetic acid	s. d. Fine Chemicals Ltd., India
Chloroacetic acid	Loba Chemie, India
Chlorobenzene	s.d. Fine chemicals Ltd., India
Colchicine	Loba Chemie, India
Eosin	Qualigens Fine Chemicals, India
Ethyl alcohol	Changshu Yangyuan Chemicals, China
Fetal bovine serum	s.d. Fine chemicals Ltd., India
Giemsa	s. d. Fine Chemicals Ltd., India
Haematoxylin	Qualigens Fine Chemicals, India
May-Gruenwald	Merck Chemical Ltd India
Potassium chloride	Ranbaxy, S.A.S. Nagar
Potassium phosphate monobasic	Qualigens Fine Chemicals, India
Sodium phosphate diabasic	Central Drug House, India

Preparation of solutions:

Colchicine	10 mg Colchicine was dissolved in 25 ml of distilled water, final concentration 0.4 mg colchicine/ml.
Hypotonic KCl (0.56%)	0.56 g KCl dissolved in 1,000 ml distilled water (final concentration 0.56% KCl).
Fixative (Aceto: Alcohol=1:3, v/v)	To 75 ml of methanol, 25 ml of acetic acid was mixed with continuous shaking.
Sorenson's Buffer Solution (SBS)	**A.** 5.938 g sodium phosphate (dibasic) was dissolved in 100 ml of distilled water. **B.** 4.538 g Potassium phosphate (monobasic) was dissolved in 100 ml of distilled water. 50 ml each of **A** and **B** were mixed to prepare SBS.
Scott's tap water	0.2 gm potassium bi-carbonate and 2 gm magnesium sulphate were dissolved in 100 ml of distilled water.
Giemsa 5%	5 ml of Giemsa was mixed to 95 ml of SBS to prepare a solution of 5% Giemsa.

Test chemicals:

Liquid chlorobenzene (99%) and crystalline chloroacetic acid (99% purity) were used as test chemicals. There properties have been listed in the Introduction (page No. 11 and 16).

Preparation of stock solutions:

The doses were selected from a published report (NTP, 1992). They were standardized during the pilot experiments carried out in our laboratory.

1. Chlorobenzene Intraperitoneal injection to test animals were given as per the LD_{50} values reported for *Rattus norvegicus*. LD_{50} (male)-165 mg / 100 gm b.wt; and LD_{50} (female)-165 mg / 100 gm b.wt.

Dozes of the following three concentrations were administered to three different experimental groups:

i) 75mg / 100g b.wt.
ii) 100mg / 100g b.wt.
iii) 125mg / 100g b.wt.

2. Chloroacetic acid Usually 100 ml stock solution containing 1mg / ml was prepared in distilled water. As reported for *Rattus norvegicus*, LD_{50} values of 1.6 mg / 100g b.wt. for males and 1.6 mg / 100 g b.wt. for females respectively, were used to inject experimental groups of rats in this study.

Dozes of the following three concentrations were used:

i) 0.8 mg / 100g b.wt.
ii) 1 mg / 100g b.wt.
iii) 1.2 mg / 100g b.wt.

Experimental Design:

Source and Maintenance of Rats:

Laboratory-bred male and female (*Rattus norvegicus*) albino rats (2n=42) 8-10 weeks old weighing 100 ± 20 g, were procured from animal laboratory house CDRI, Lucknow, India. The animals were bred and maintained under standard laboratory conditions. Commercial pellet diet and water were given to them *ad libitum*. For both the test chemicals, 108 rats were divided into three experimental groups. A group of three rats were used for each concentration, and 2 as per the durations indicated below corresponding tables/bar diagrams.

The control groups were injected with distilled water; each control group also consisted of 9 rats accounting to a total of 36 animals.

Treatment of Animals by Test Compounds:

12, 24 and 48 h following the injection, three rats per duration / per concentration were sacrificed and immediately dissected to obtain bone marrow for cytogenetic preparations. The explained design for chromosomal aberration (CA) and micronucleus test (MNT) follows:

120 minutes (min) prior to killing, colchicine (4 mg/kg, i.p.) was administered to the animals. The time of sacrifice was decided on preliminary observations for scoring as optimal aberrations. The slides of bone marrow cells were prepared and stained according to the routine hypotonic-acetic acid-methanol-flame drying-Giemsa schedule for metaphase plate analysis (Preston *et al.*, 1987).

Slide preparation:

Briefly, both the femur of each animals were removed and bone marrow cells harvested by aspiration with a syringe containing 0.56% (0.075M) KCl. The cells were treated with hypotonic solution 0.56% KCl at 37°C for 30 min. The cells were then centrifuged at 1,500 rpm for 10 min. This was followed by fixation in glacial acetic acid: methanol (1:3). The cells were then prepared for microscopial examination by adding 3-4 drops of cell suspension to pre-cleaned and pre-chilled (in ethanol-dipped) microscopic slides. These slides were air-dried and stained with Giemsa (5%) for 30 minutes.

Cytogenetic Testing Parameters:

The parameters used for cytogenetic tests were:

a. Chromosomal Aberrations (CA)

b. Micronucleus Test (MNT)

a. Chromosomal Aberration Assay

At least hundred well spread intact metaphases were scored per animal at a magnification of 100X with oil Emmersion. The type of chromosomal aberrations included chromatid and chromosome breaks and chromosomal rearrangements.

i) Cytogenetic methods or procedures

Chromosome preparation from rat bone marrow metaphase was done according to the protocol of Preston *et al.*, 1987. A flow chart is given below:

Chlorobenzene or Chloroacetic acids were injected intraperitoneally (i.p.) with several concentrations.

↓

Before two hours of sacrifice, Colchicine was injected (i.p.) to treated rats at a concentration of 4mg / kg b.wt. (Krishna *et al.*, 1985).

↓

Femurs were collected from sacrificed rats and cleaned by distilled water.

↓

Both ends of femur were cut by scissor and bone marrow flushed out with 0.56% KCl.

↓

Collected bone marrow was incubated at 37°C for 30 min and centrifuged at 1,500 rpm for 10 min.

↓

Pellet was resuspended in freshly prepared fixative.

↓

Following centrifugation at 1,000 rpm for 5 min, the supernatant was discarded and centrifuged again. This step was repeated 2 more times. The supernatant was discarded and pellets saved and resuspended in 1ml of the above aceto-alcohol (acetic acid: methanol) fixative. Then it was dropped on pre-cleaned and pre-chilled ethanol-dipped slides.

↓

Slides were flame dried.

↓

Staining was done with Giemsa 5% in SBS.

↓

Followed by cleaning with Xylene and

↓

Mounted with D.P.X. and

Observations were made at 100X under oil Emmersion.

ii) Microscopic analysis

The slides were screened for metaphases initially at low magnification (45X) and subsequently at high magnification of 100X. Best preparations, which permitted the desired analysis of mitotic cells were selected on the following basis:

1. Well spread metaphases with 2n=42, which permitted the analysis of fully aligned chromatids.
2. There should not be centromere splitting.
3. Extensive chromosome overlapping lacking and
4. With best fixation quality.

Chromosomal aberrations were classified following criteria of international nomenclature (1978), according to which, they were categorized into two major types; structural and numerical. The structural types of aberrations were studied further under two sub-types chromatid and chromosome-type aberrations. Chromatid type aberrations involved one chromatid only.

Following criteria were used to count chromosomal aberrations:

1. Chromatid breaks (B) having true discontinuities with clearly dislocated fragment, where fragment almost remained associated with chromosome of origin.
2. Chromatid gap (G), where an achromatic lesion was formed with smaller than the width of one chromatid.
3. Chromosomal type of aberration involved both the chromatids of a chromosome at identical site or loci and comprised of abnormalities like gap (G') or achromatic lesion, having non-staining region not greater than the diameter of chromatid.
4. In cases where break or (terminal deletion) involved only one chromosome; chromosomal exchanges where two or more lesions in the same or different chromosomes were observed.
5. On the other hand, multiple aberrations (MA) comprised of rings, acentric, dicentrics, stickiness and pulverization. Another categories of multiple aberration was noted as aneuploidy where the chromosome number of a cell deviated from a multiple haploid set (21 in present case).

b. Micronucleus Test Assay

Test animals were sacrificed after 12, 24 and 48 h of treatment with three different concentration of chlorobenzene and chloroacetic acid, respectively. On the basis of some previous reports (Mohtashamipur *et al.*, 1987), the time of peak response of micronuclei induction was selected and animals were sacrificed.

Slides of each MNT preparation/dose were made in duplicate. One of them was processed according to Schmid (1975), while the duplicate slide was stained following the protocol of Pascoe and Gatehouse, 1986.

Scheme of Schmid (1975), is stepwise summarized below:

Chlorobenzene and Chloroacetic acid in a decided dose was injected intraperitoneally (i.p.) into the test animals

↓

Animals were sacrificed at different durations

↓

Both femora were dissected out and their marrow cells were flushed out with 1ml of fetal bovine serum (FBS) by pipetting it for several times

↓

Aliquot of cell suspension was centrifuged at 1,000 rpm for 5 min.

↓

Finally, cell pellet resuspended in a small amount of FBS and smeared on a clean glass slide

↓

Dried over night

↓

Fixation was performed in 100% methanol for 5 min.

↓

Fixed slides were stained in May-Gruenwald and Giemsa 5% for 25-30 min.

↓

Cleaning was done by Xylene

↓

Mounting with D.P.X

All preparation were labeled and observed immediately.

II) Another protocol for MNT

Pascoe and Gatehouse (1986), slightly modified the method of Schmid (1975) All the steps were the same as originally described by Schmid, except that staining was carried out in Haematoxylin and eosin, respectively.

ii) Microscopic analysis

Labelled slides of controls as well as those of treatments were scored by a single observer and screened for micronuclei under the magnification 100X. The right area on the slide was selected for scoring where the erythrocytes were well separated, not folded and could clearly be discriminated. Well-stained slides were chosen for the discrimination between polychromatic erythrocytes (PCE_S) and normochromatic erythrocytes (NCE_S). PCE_S were purple to blue, while NCE_S were orange to red.

Statistical analysis:

Statistical evaluation was done by applying Students' *t*-test, and values were considered significant at $p < 0.05$.

RESULTS

Figures 1 and 2, which show the metabolic activation mechanism of Chloroacetic acid (CAA) and Chlorobenzene (CB) have been added under "Introduction" (page No.12 and 17). External morphology of the rat, *Rattus norvegicus* used in the present study is shown in Fig. 3 (A). The standard karyotype of male and female *R. norvegicus* are respectively shown in Fig. 3 B and C, which is from the internet. The original photomicrograph of metaphase chromosome plate taken during the present study is shown in Fig. 6 at a magnification of 45×10x, which confirms that the animals used had the same karyotype.

Rats were treated in three different dozes of both the chemicals, and the dozes have been depicted as D_1, D_2 and D_3 in the increasing order of the concentrations. For per 100 g body weight D_1 was 75 mg for CB and 80 mg for CAA; D_2 was 100 mg for the both chemicals, and D_3 was 125 mg for CB and 120 mg for CAA.

Release of micronuclei (MN) as mutagenic effects of CAA and CB using various parameters have been summarized in Table 1 and 2. The results demonstrate microscopic analysis of changes in frequency

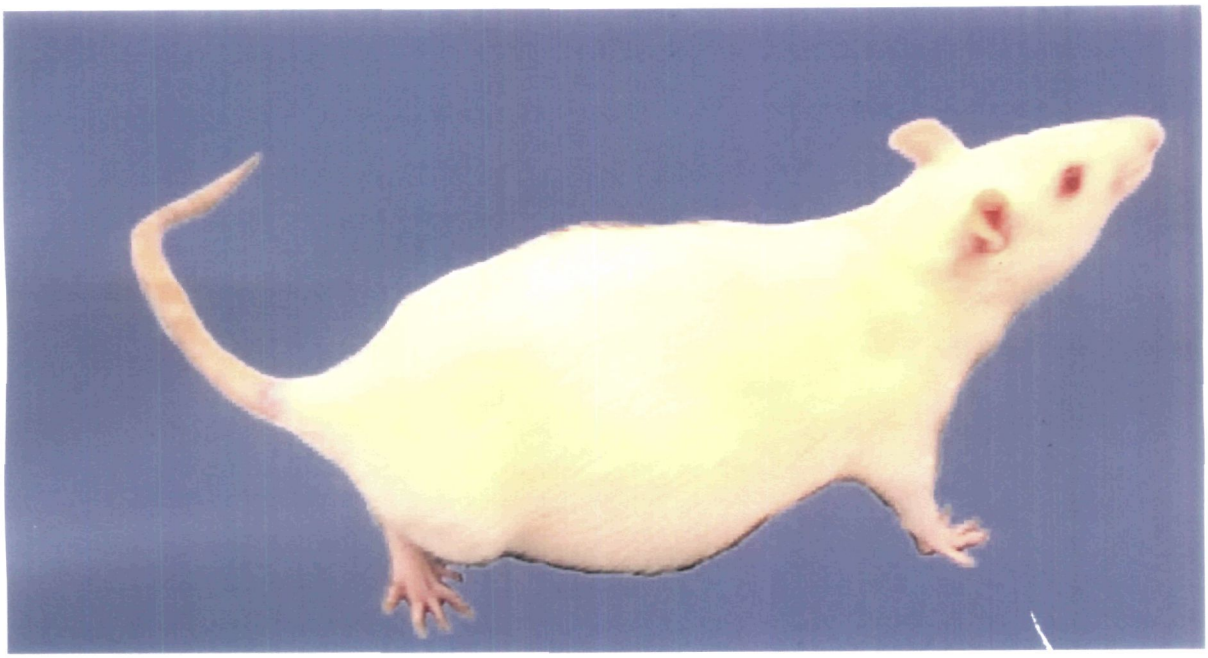
of MN induced by various concentrations of CAA and CB in rat bone-marrow cells. Intraperitoneal administration to both CAA and CB displayed an induction of MN that was significant at $p< 0.05$. Chemically induced formation of MN was detectable at 12, 24, and 48 h treatment, respectively. Erythrocytes take different stains depending on their age. Young erythrocytes appear bluish and are termed as polychromatic (Schmid, 1975), whereas normochromatic erythrocytes or aged ones take red colour in the samples of 12, 24 and 48 hours duration.

Mutagenic effects of CAA and CB were evaluated at 12, 24 and 48 h in rat-group that received different dozes. The dozes (D_1-D_3) of both chemicals were as detailed above in the first paragraph. Typical results (plates) of micronucleated polychromatic erythrocytes (MNPCEs) formation at D_1, D_2 and D_3 of CAA and CB are shown in Fig. 4-5. Table-1 and 2 are based on the data collected by counting MN from plates similar to those shown in Fig.4-5. At all dose levels, the frequency of micronuclei in polychromatic erythrocytes (PCE_S) induced by CAA was found consistently elevated. The maximum value of 4.47±0.5 was recorded at 24 h of D_3 as compared to that of the controls (1.00±0.3), while the maximum value for CAA treated

rats was 4.40±0.3. There was a dose (D_3) and duration dependent increase in the formation of $MNPCE_S$, which was maximum at 24 h of the treatment with both chemicals.

The presented data in Tables-3 and 4 indicate that CAA and CB are both cytotoxic and clastogenic. The dose and time dependent total chromosomal aberrations are summarized in Table-3. As shown in Table-3, there was a significant increase in aberrations at 24 h (Table-3). However, extending the CAA treatment for another 24 h (*i.e.* for a total of 48 h) resulted in definite decrease in total aberrations. Results presented in Table-4 and histogram based on the data shown in Fig. 11, reveal that the effect of CB on *R. norvegicus* was similar to that observed for CAA. Highest aberrations in either case were recorded at 24 h, while a clear decrease was observed at 48 h. A maximum of 4.55±0.3 frequency of aberration was recorded in CB treated groups at 24 h of D_3 as compared to 4.33±0.2 of CAA, showing relative effectiveness. No significant difference was observed in controls given normal (tap water) or the solvent (distilled water) in which CAA and CB were dissolved.

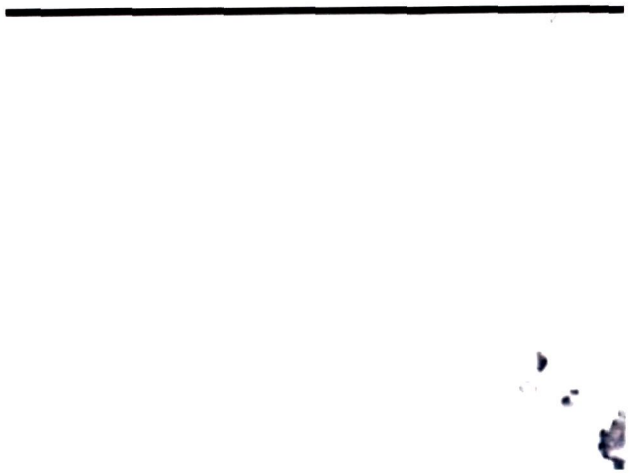
Fig. 6 (plate) shows a normal metaphase plate of the rat bone marrow, while Fig. 7 (plates, 1-5) illustrates the changes in morphology following treatment with clastogen CB and CAA, respectively. As shown on plates of Fig. 7, these agents caused chromosome damage, which includes chromosomal, and chromatid breakage, gaps and exchanges. The data obtained from counting aberrations from such plates is summarized in Table 3 and 4, which again demonstrates that the maximum changes occur at 24 h and decline thereafter.



A

B C

Fig.3: **A**-The photograph of female *Rattus norvegicus*; **B** and **C**- The standard karyotype of male and female *Rattus norvegicus* (Committee, 1973).



1

2

3

Figure 4. Plates 1-3 showing the normal polychromatic erythrocytes in bone marrow cells of *Rattus norvegicus*.

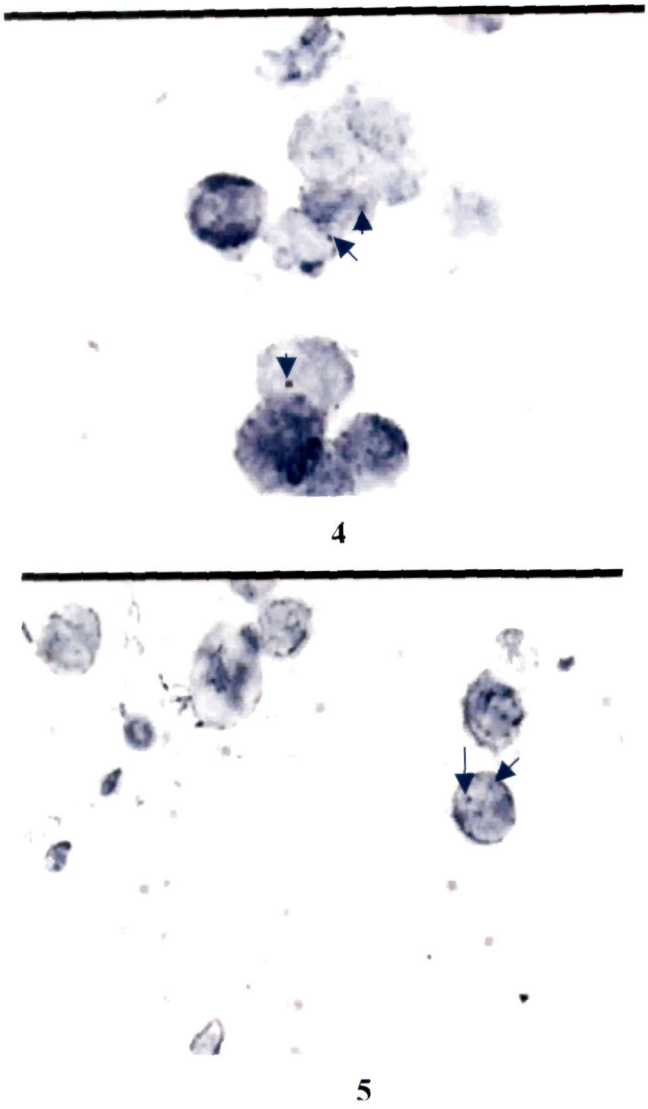


Figure 5. Plates -4and 5showing micronucleus as indicated by arrow in polychromatic erythrocytes of *Rattus norvegicus*.

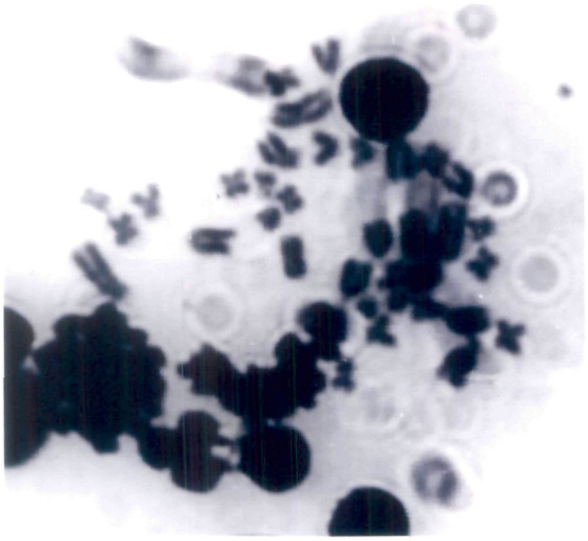


Figure 6. Metaphase chromosome plate showing the normal chromosomes in bone marrow cells of *Rattus norvegicus*.

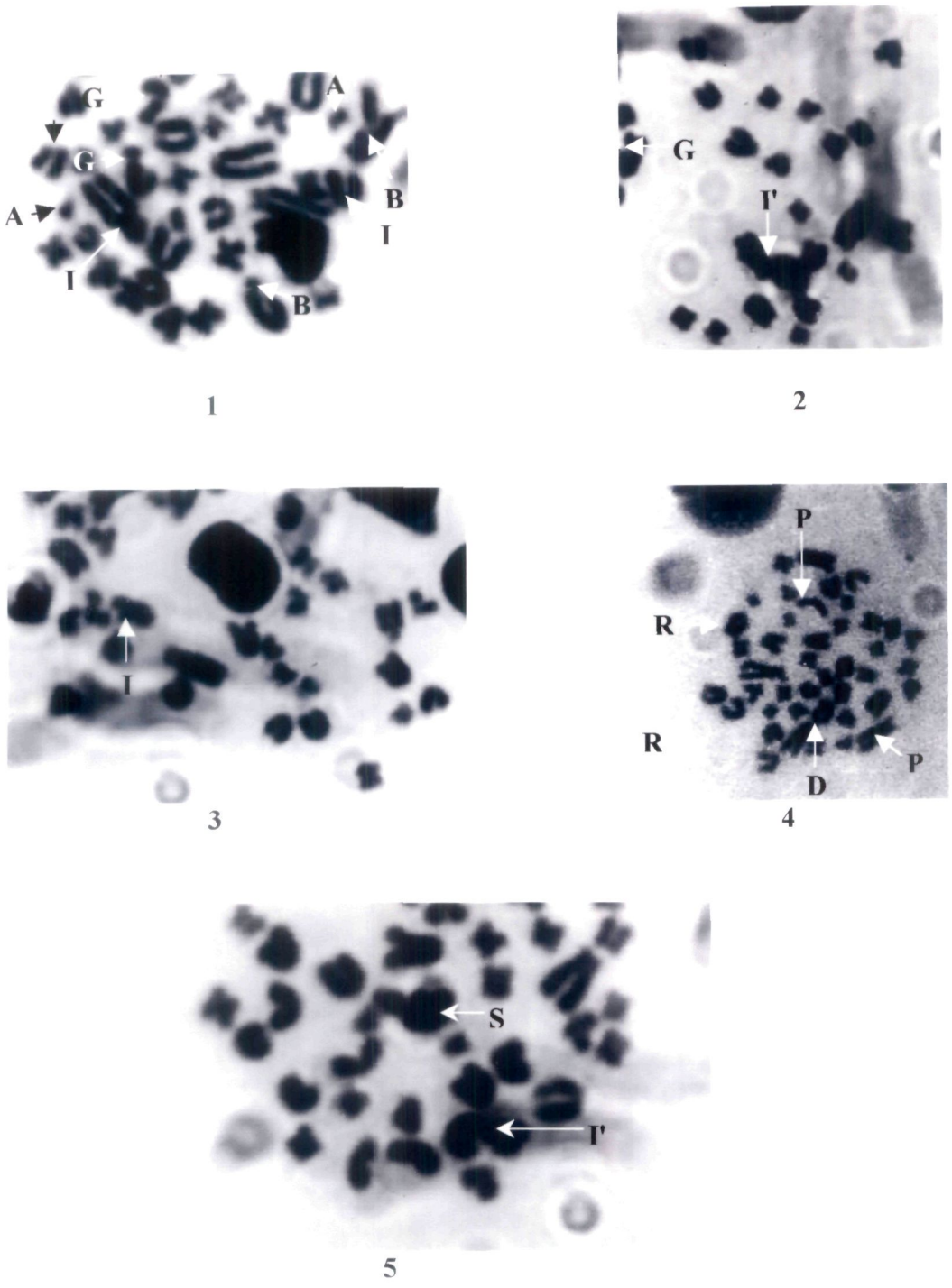


Figure 7. Metaphase chromosome plates 1-5 showing the chromosomal aberrations **A-** Acentric chromosome; **B-**Chromatid break; **D-** Double fragment; **G-** Chromatid gap; **I-** chromatid exchange; **I'-** Chromosomal exchange or rearrangement **P-** pulverization **R-** Ring chromosome; **S-** stickiness;

Table: 1. Frequency of micronuclei in bone marrow cells of *Rattus norvegicus* treated with Chloroacetic acid.

Treatment	Duration (Hrs.)	Concentration (Mg/100gm b.wt.)	Total number of cells analyzed	No. of PCEs analyzed	No. of MNNCEs	No. of MNPCEs	No. of micronuclei per 1000 PCEs
Control	-	-	6000	2001	1	2	0.99±0.1
Chloroacetic-	12	0.8	6000	2929	3	6	2.04±0.2
Acid	24	0.8	6000	2942	4	9	3.05±0.3
"	48	0.8	6000	2839	2	6	2.11±0.8
Control	-	-	6000	2002	1	2	0.99±0.1
Chloroacetic-	12	1.0	6000	2908	2	7	2.40±0.6
Acid	24	1.0	6000	2962	4	11	3.71±0.6
"	48	1.0	6000	2933	3	8	2.72±0.8
Control	-	-	6000	2000	1	2	0.99±0.1
Chloroacetic-	12	1.2	6000	2933	3	8	2.72±0.5
Acid	24	1.2	6000	2952	4	13	4.40±0.2*
"	48	1.2	6000	2916	2	9	3.08±0.5

PCEs, polychromatic erythrocytes; **NCEs**, normochromatic erythrocytes.
Values are from three rats in each group and represent **mean ±SD** of per 1000 PCEs.
Values set up at (**$p<0.05$**). * - Significant value.

Table: 2. Frequency of micronuclei in bone marrow cells of *Rattus norvegicus* treated with chlorobenzene.

Treatment	Duration (Hrs.)	Dose (Mg/100gm b.wt.)	Total number of cells analyzed	No. of PCEs analyzed	No. of MNNCEs	No. of MNPCEs	No. of micronuclei per 1000 PCEs
Control	-	-	6000	2001	1	2	0.99±0.1
Chlorobenzene	12	75	6000	2901	2	6	2.06±0.8
"	24	75	6000	2938	4	9	3.06±0.5
"	48	75	6000	2896	2	7	2.41±0.5
Control	-	-	6000	2002	1	2	0.99±0.1
Chlorobenzene	12	100	6000	2869	3	7	2.43±0.5
"	24	100	6000	2957	4	11	3.71±0.5
"	48	100	6000	2925	3	8	2.73±0.9
Control	-	-	6000	2000	1	2	0.99±0.1
Chlorobenzene	12	125	6000	2918	3	8	2.74±0.8
"	24	125	6000	2951	4	13	4.47±0.3*
"	48	125	6000	2924	2	9	3.07±0.3

PCEs, polychromatic erythrocytes; **NCEs**, normochromatic erythrocytes.
Values are from three rats in each group and represent **mean ±SD** of per 1000 PCEs.
Values set up at (**$p<0.05$**). * - Significant value.

Table: 3. Chloroacetic acid- induced chromosomal aberration in bone marrow cells of *Rattus norvegicus*.

Treatment	Dose (Mg / 100gm b.wt.)	Duration (Hrs.)	Chromatid aberration		Chromosomal aberration			Total aberration (%)	
			B	G	B'	G'	MA'	Without gaps	With gaps
Control	-	-	-	1	-	-	1	0.3	0.66±0.1
Chloroacetic-	0.8	12	1	-	0	1	4	1.6	2.00±0.6
Acid	0.8	24	2	1	1	2	3	2.0	3.00±0.5
"	0.8	48	1	1	1	1	3	1.6	2.33±0.3
Control	-	-	-	1	-	-	1	0.6	0.66±0.1
Chloroacetic-	1.0	12	2	1	2	1	1	1.6	2.33±0.3
Acid	1.0	24	3	2	2	2	2	2.3	3.70±1.3
"	1.0	48	1	2	2	1	3	2.0	2.66±0.4
Control	-	-	-	1	-	-	1	0.3	0.66±0.1
Chloroacetic-	1.2	12	2	1	1	1	3	2.0	2.66±0.3
Acid	1.2	24	3	2	3	1	4	3.3	4.33±0.6*
"	1.2	48	1	2	2	1	2	1.6	2.77±0.4

Abbreviations: B- chromatid breaks, **G** - chromatid gaps, **MA** - multiple aberration; **B'** - chromosomal breaks, **G'** - chromosomal gaps, **MA'** - chromosomal multiple aberration.
Values are from three rats in each group and the last column represent **mean ±SD** of sum of aberration with gaps.
Values set up at (**$p<0.05$**). * - Significant value.

Table: 4. Chlorobenzene - induced chromosomal aberration in bone marrow cells of *Rattus norvegicus*.

Treatment	Dose (Mg / 100gm b.wt.)	Duration (Hrs.)	Chromatid aberration		Chromosomal aberration			Total aberration (%)	
			B	G	B'	G'	MA'	Without gaps	With gaps
Control	-	-	-	1	-	-	1	0.3	0.66±0.1
Chlorobenzene	75	12	2	1	1	1	2	1.6	2.33±0.2
"	75	24	2	2	2	1	3	2.3	3.33±0.3
"	75	48	1	1	1	1	3	1.6	2.44±0.3
Control	-	-	1	-	-	-	1	0.3	0.66±0.1
Chlorobenzene	100	12	2	1	2	1	2	2.0	2.50±0.3
"	100	24	3	2	2	1	4	3.0	4.00±0.2
"	100	48	2	2	2	0	2	2.0	2.66±0.8
Control	-	-	1	-	1	-	-	0.6	0.66±0.1
Chlorobenzene	125	12	2	2	2	1	2	2.0	2.81±0.8
"	125	24	4	2	3	2	2	3.0	4.55±0.5*
"	125	48	2	2	1	1	2	1.6	2.89±0.7

Abbreviations: **B**- chromatid breaks, **G** - chromatid gaps, **MA** - multiple aberration; **B'** - chromosomal breaks, **G'** - chromosomal gaps, **MA'** - chromosomal multiple aberration.

Values are from three rats in each group and the last column represent **mean ±SD** of sum of aberration with gaps. Values set up at (**$p<0.05$**). * - Significant value.

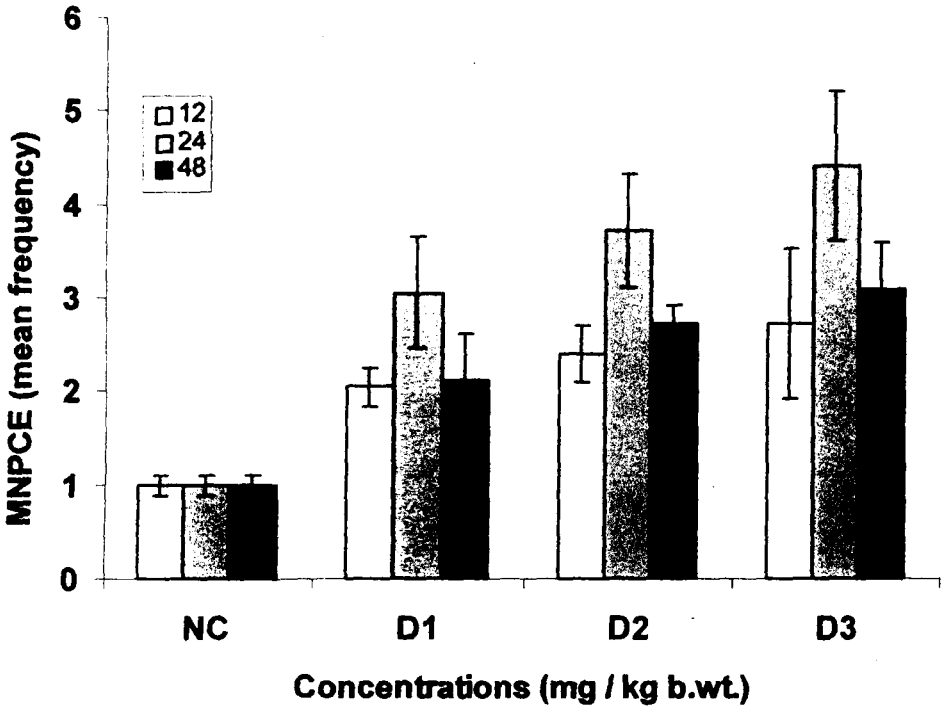


Figure 8. Dose-duration dependent profile of Micronucleated Polychromatic Erythrocytes ($MNPCE_S$) by Chloroacetic acid (CAA) in *Rattus norvegicus*.

NC, Normal Control, Distilled Water; D1, Dose 80mg / kg; D2, Dose 100mg / kg; D3, Dose 120mg / kg. In the bars vertical lines show the values of Mean ±SD.

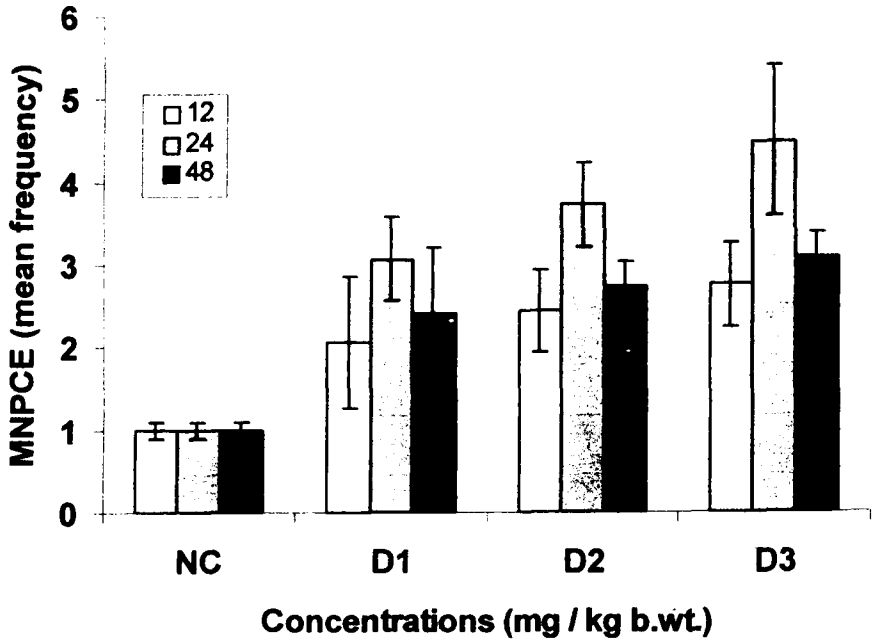


Figure 9. Dose-duration dependent profile of Micronucleated Polychromatic Erythrocytes ($MNPCE_S$) by Chlorobenzene (CB) in *Rattus norvegicus*

NC, Normal Control, Distilled Water; D1, Dose 75 mg / kg; D2, Dose 100 mg / kg; D3, Dose 125 mg / kg. Vertical lines are Mean ±SD.

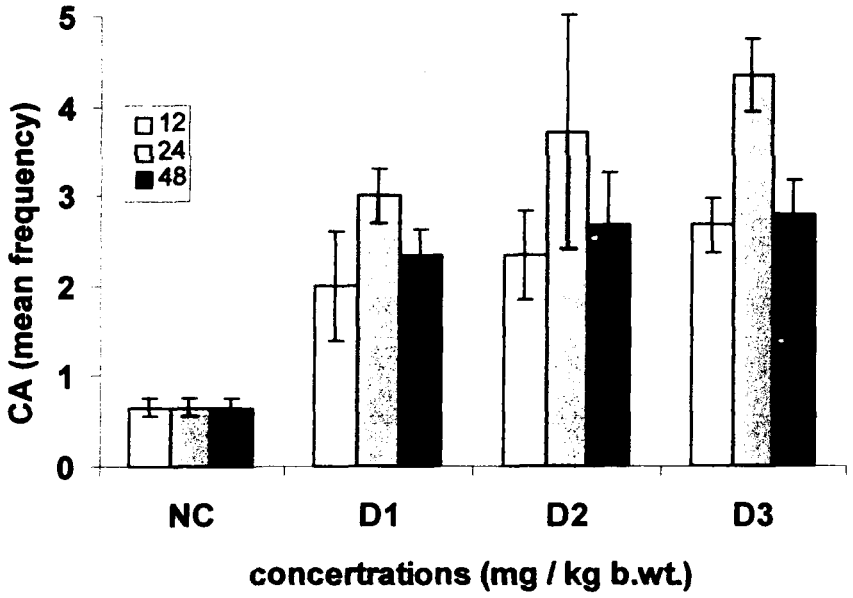


Figure 10: Multiple concentration and duration related profiles of Chromosomal Aberration (CA) by Chloroacetic acid (CAA) in *Rattus norvegicus*

NC, Normal Control, Distilled Water; D1, Dose 80mg / kg; D2, Dose 100mg / kg; D3, Dose 120mg / kg. Values are Mean ±SD.

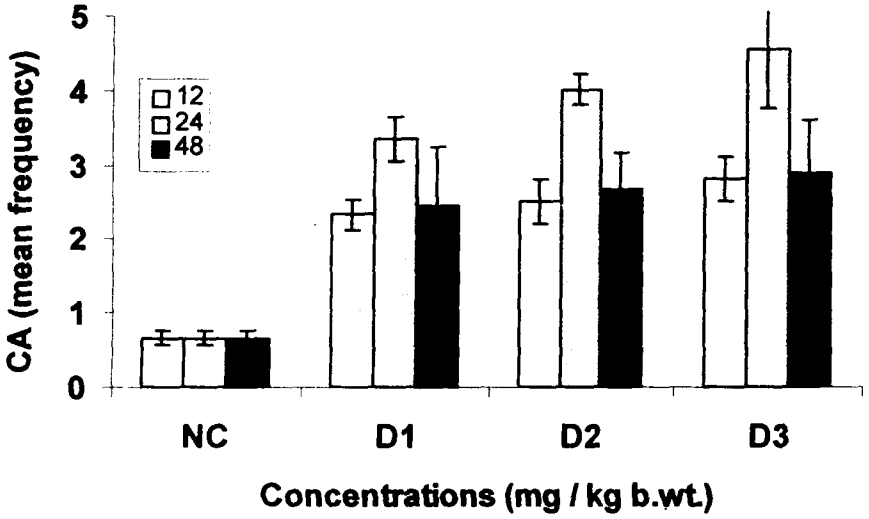


Figure 11: Multiple concentration and duration related profiles of Chromosomal Aberration (CA) by Chlorobenzene (CB) in *Rattus norvegicus*

NC, Normal Control, Distilled Water; D1, Dose 75mg / kg; D2, Dose 100mg / kg; D3, Dose 125mg / kg. Values are Mean ±SD.

DISCUSSION

In view of the harmful effects of pesticides and food additives, which are being consistently recorded, it is essential that toxic effects of these chemicals are evaluated under variety of conditions. This study deals with two mutagens, chloroacetic acid (CAA) which is used as herbicide and for treatment of plantar warts, while chlorobenzene (CB) is an industrial solvent, apart from having applications in pharmaceutical industry. Both of these compounds are used as preservatives and drying agent for curing hay. CB alone has wide applications as a degreasing solvent, in dry cleaning industry and in manufacturing pesticides, resins, dyes, perfumes and the synthesis of organochlorine pesticides (U.S. EPA, 1988). As for hazards posed to human beings, at least two deaths have been reported due to exposure to CAA (Zeldenrust, 1951; Mann, 1969). CB can also enter human systems, while part of it may be metabolized, some of it remains unchanged and exhaled out in breath (Williams, 1959).

Though new and sophisticated techniques are being developed, several conventional techniques still provide useful information on toxicity of suspected compounds. Two of such techniques, which have

been widely used, are: **i)** *in vivo* bone marrow chromosomal aberrations (CAs) and, **ii)** micronucleus (MN) assay. In the present study the mutagenic potential of CAA and CB has been analyzed in bone marrow cells of *Rattus norvegicus* employing these techniques; *i.e.* CA and MN assays, with the polychromatic erythrocytes being part of the latter assay.

Only few studies have focused attention on the mechanism of micronucleus formation. Evans *et al.* (1959) showed that when the root tips of *Vicia faba* were subjected to fast neutrons and gamma rays exposure, induction of chromosomal aberrations resulted in micronuclei formation. Schmid (1976) showed that micronuclei are formed due to chromosomal breaks and spindle inhibition. It is generally accepted that clastogenic mutagen induce micronuclei formation which are break-away part of chromosome fragments, or of lagging chromosomes (Schmid, 1975). Heddle and Carrano (1977) have worked out theoretical aspects of the relationship between chromosomal aberrations and micronucleus formation.

CAA and CB, in the concentrations given to rats during present investigations caused chromatid/chromosomal breaks, gaps, exchanges, multiple aberrations and micronuclei formation in the bone marrow cells

of *Rattus norvegicus*. It is evident that in addition to an apparent interference during chromosome condensation due to chromosome breakage, fragmentation and disintegration, which demonstrates the clastogenic potential, both of these chemicals also cause spindle poisoning (Sharma *et al.*, 2000).

In the present study after treating *Rattus norvegicus* with CAA and CB, a dose and durations dependent increase in CA and MN formation of polychromatic erythrocytes were recorded. Our data demonstrate that CB is more damaging mutagen than CAA, since the maximum frequency of chromosomal aberrations with CB had the values of 4.55±0.5 as compared to 4.33±0.6 with CAA. Similar conclusion can be made from the data on micronuclei assay as the maximum value of 4.47±0.3 was obtained for CB, while the value for CAA was 4.40±0.2. In both cases, significant differences were observed at 24 h only. Rats treated with both of these chemicals showed a conspicuous decrease in aberrant type cells and micronuclei at 48 h. Such a decrease has been attributed to elimination of metabolites from the body, repair of damaged genetic material, removal of cell chromosomes with damaged genetic material, or caused by reduction in the level of test compound due to its

conversion to other products (Tates and Natarajan, 1976; Bhunya and Behera, 1987).

As per our results, CAA is more mutagenic than the related compound TCA, since CAA caused significant damage in bone marrow cells of *R. norvegicus* at a concentration of 1.2 mg/100 gm b.wt., *i.e.*12 mg/kg, while 500 mg/kg of TCA was required in case of mice (Bhunya and Behera, 1987). In other words, taking the parameters used here, CAA is >40 times more mutagenic than TCA. The results also show that CAA and CB cause rapid decrease at 48 h in the values of CAs and MN in bone marrow cells following an initial increase observed at 24 h of treatment, which may be due to the reasons outlined in the preceding paragraph.

Present study suggests that CB is clastogenic to *R. norvegicus*. A number of reports do not support the genotoxic potential of CB. These studies employed a wide range of experimental techniques, but failed to show any genotoxicity of the CB (Shimuzu *et al.*, 1983; Loveday *et al.*, 1989; Williams *et al.*, 1989; Hellman, 1992). However, evidence to contrary also exists. Metabolites of CB can bind to nucleic acid both *in vivo* and *in vitro* (Grilli *et al.*, 1985; Prodi *et al.*, 1986; Colacci *et al.*,

1990) causing damage to DNA, which confirms that at high doses (3×750mg/kg) of CB is genotoxic to rats and mice (Vaghef and Helman, 1995). Increased frequency of gene mutations in peripheral lymphocytes has been recorded in human (Major *et al.*, 1993), who were exposed to CB for several years.

Sub lethal concentration at 70% LD_{50} value of CB has also been reported to induce a dose-related increase in the number of MNPCEs in the bone marrow of mice at 30 h duration, indicating a genotoxic and clastogenic potential of this compound (Mohtashamipur *et al.*, 1987). It is to emphasize that the report of Mohtashamipur *et al* (1987) described results on *in vitro* study, whereas the present work is an *in vivo* investigation. Another report suggested that, maximum chromosomal damage of CB was showed at 24 h on root tip cells of *Vicia faba* (Liu *et al.*, 2003). The fact that maximum chromosomal damage is recorded at 24 h in plants as well as in animals suggests that there are certain common elements in the mechanism of interaction of CB with chromosome/ DNA. In the present study, 75% of the reported LD_{50} dose of CB affected the bone marrow cells of rats displaying a significant chromosomal damage at 24 h.

Our results, therefore, support mutagenic potential of CB rather than a benign interaction between this compound and chromosomes or DNA.

SUMMARY

Widely used herbicides and chemical intermediates, chloroacetic acid (CAA) and chlorobenzene (CB) have been evaluated in terms of the extent of genetic damage. Cytogenetic parameters used to monitor their effect are:

(i) Chromosomal aberrations such as breaks, gaps, exchanges, ring, translocations and multiple aberrations and

(ii) Micronucleus tests on bone marrow cells of rat (albino), *Rattus norvegicus*.

To determine LD_{50} values, three sub-lethal concentrations of each chemical were administered for 3 different time intervals. The results lead to following inferences:

(1) While chromatid type aberrations were more abundant, both of the chemicals induced chromatid as well as chromosome aberrations. Thus chromatid type gaps were frequently observed in concentrations of CB and CAA.

(2) Clastogenic potential of CB and CAA was demonstrated by the maximum frequency of chromosomal aberrations with the values of 4.55±0.5 and 4.33±0.6, respectively.

(3) The maximum impact of chemical CB and CAA on rat bone marrow was observed at 24 h of treatment. The values were statistically significant in comparison with those obtained for other durations.

(4) Upon prolonging *in vivo* treatment with either of the compounds to 48 h, a significant decrease in clastogenic damage was recorded. No significant increase could be observed in normal control and that given solvent only.

(5) Micronucleus (MN) assay supported the observed dose- and time-dependence of chromosomal aberrations, as mentioned above for CAA and CB. MN value was 4.47±0.3 for CB as compared to 4.40±0.2 for CAA, indicating higher mutagenicity of CB.

(6) So far as micronucleated polychromatic erythrocytes (MNPCEs) are concerned, dose- and time-dependent effects of CB and CAA were parallel to those observed in MN data.

(7) According to the above observations CAA and CB are mild mutagens, and if susceptibility of *Rattus norvegicus* to them is taken into account, possible exposure of other mammals including humans to these compounds should be minimized.

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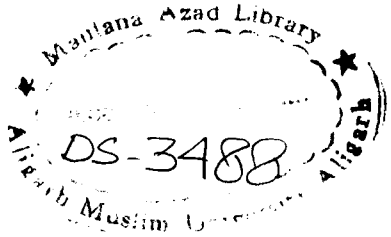

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